# A LABORATORY MANUAL ON ABNORMAL HAEMOGLOBINS

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# ABNORMAL HAEMOGLOBINS

*prepared under the direction of*

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*and*

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*for*

THE COUNCIL FOR INTERNATIONAL ORGANIZATIONS OF MEDICAL SCIENCES

*and the*

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# PREFACE

The present brief Manual on Techniques for Studying Abnormal Haemoglobins was prepared as a sequel to the Training Course held in September 1957 under the auspices of the Unesco Middle East Science Co-operation Office  This course followed the international symposium on 'Abnormal Haemoglobins' organized at the University of Istanbul by the Council for International Organizations of Medical Sciences (CIOMS) with the help of a grant from the Rockefeller Foundation

This manual is the work of Professor J H P. Jonxis of the University of Groningen, who served as scientific secretary of the symposium, and of his associate, Dr T H J. Huisman  A number of investigators who took part in the symposium gave helpful advice  The manual is being issued as a joint Unesco/CIOMS publication to accompany the proceedings of the symposium [1]  It is hoped that it will be of service to various workers in this field

The subject of Abnormal Haemoglobins is a phase of molecular biology which has had a great development in recent years and its study in years to come, particularly on a broad regional basis, promises to contribute much to an understanding of the genetic mechanism responsible for the production of protein molecules, as indeed to such basic problems as the nature of the gene and molecular reproduction generally  It is essential to an elucidation of the underlying causes of various pathological conditions  Moreover, the question of the geographical distribution of different normal and aberrant forms of haemoglobin is, like that of the blood groups, of the greatest interest to physical anthropology in its bearing on the interrelationships of different elements of the human population  If this manual serves in any degree to stimulate and guide field observations on the occurrence of abnormal haemoglobins, particularly in Asia and Africa where so many new discoveries have recently been made, it will have more than served its purpose

JEFFRIES WYMAN
*Director,*
*Unesco Middle East Science*
*Co-operation Office.*

Cairo, *February 13th,* 1958

[1] *Abnormal Haemoglobins  A Symposium organized by the Council for International Organizations of Medical Sciences  Edited under the direction of J H P Jonxis.* 1958  Blackwell Scientific Publications, Oxford

# THE HAEMOGLOBINOPATHIES

Since Pauling's discovery in 1949 that the haemoglobin of patients suffering from sickle cell anaemia differs from normal haemoglobin, there has been rapid progress in the field of the hereditary anaemias. An increasing number of forms of hereditary anaemias that are often accompanied by the presence of one or more abnormal haemoglobins has been described

The name of 'haemoglobinopathies' has been proposed for this group of diseases with which thalassaemia (also known as Cooley's anaemia) is to be included.

This group of diseases is important to physicians of many countries Indeed, in various parts of the world the number of carriers of one of these hereditary abnormalities is relatively high In some cases, as in sickle cell anaemia and Cooley's anaemia, the carriers enjoy a certain protection against malaria which is especially important in the younger age groups This may explain the high frequency of these abnormalities in certain regions where malaria is prevalent A study of the occurrence of these diseases is also of great importance to anthropologists since different abnormal haemoglobins seem to have had their origin as gene-mutations in different regions and their occurrence, therefore, has a bearing on interrelationships of different elements of the world population

Two forms of each abnormal condition can be differentiated. They differ in severity

(1) When both parents transmit the same abnormality to their offspring — in other words when the child receives abnormal genes from both parents — the condition in the child represents the homozygous state In the case of the sickle cell gene or the thalassaemia gene the homozygous condition is referred to as 'sickle cell anaemia' or 'thalassaemia major' (Cooley's anaemia)

(2) When only one parent transmits an abnormality to his offspring the child represents the heterozygous state and one refers to the condition as 'sickle cell trait' or 'thalassaemia minor' (thalassaemia trait)

As a general rule the homozygous state is more severe than the heterozygous state.

Individuals who are heterozygous for two or even three of these conditions have been described In general their health is somewhat better than that of homozygous patients with sickle cell anaemia or thalassaemia major.

### IMPORTANCE OF THE HAEMOGLOBINOPATHIES

Children born with sickle cell anaemia are often so badly handicapped that they die before they reach adulthood The same is true of thalassaemia major. The homozygous carriers of other abnormal haemoglobins are less severely affected and may even show no symptoms at all. In these cases they are detected in surveys or during the investigation of the family of a patient suffering from an anaemia

The study of these haemoglobinopathies is important for the following reasons

(1) It is necessary to distinguish these hereditary anaemias from those due to iron deficiency and those caused by malaria or intestinal parasites.

(2) When one or both parents are heterozygous for one of these diseases it is likely that the abnormality will occur in the offspring

(3) As mentioned previously, the carriers of the sickle cell and Cooley genes enjoy a certain protection against malaria, it is therefore of importance to determine the percentage of carriers of the sickle cell and thalassaemia genes in areas where malaria is common

(4) Low values of the haemoglobin concentration found in surveys are often caused by iron deficiencies, malaria or intestinal parasites However, one should take into consideration that they might be caused by the presence of certain haemoglobinopathies in a heterozygous condition

(5) In many parts of the world individuals heterozygous for one of these haemoglobinopathies are common North and Central Africa, Italy, Greece, the Middle East countries, India, Pakistan, Burma, Thailand, Indonesia and those parts of the world where there are immigrants from these countries. Physicians and medical officers in these areas should be increasingly on the look-out for these conditions

### BIOCHEMICAL CONSIDERATIONS

The haemoglobin molecule is composed of two moieties. One moiety is an iron-containing pigment — a ferroporphyrin — called 'haem'.

The other moiety is a protein called 'globin'. In fact, four haems are attached to the protein to give 'haemoglobin'.

The chief function of haemoglobin is to transport oxygen, and this reversible oxygenation is the function of the 'haem moiety' in combination with the globin.

Whereas the nature of the haem is the same for all higher animals, that of the globin varies from animal to animal The differences between the various haemoglobins found in man are much smaller than those encountered when different species are compared.

In normal man there are two physiological haemoglobins which vary in their globin moiety The haemoglobin of the human foetus *in utero* differs in many aspects from that of the adult; in particular it is far more resistant to denaturation by alkali In later months of pregnancy haemoglobin of the adult type begins to be synthesized so that, at birth, the blood of the new-born infant contains about 80% of haemoglobin of the foetal type, the balance being of the adult type After birth the concentration of foetal haemoglobin falls progressively until at the age of six months there is less than 5% left Should haemoglobin of the foetal type be found in appreciable amounts after the age of six months, some abnormality should be suspected

The two physiological haemoglobins are called respectively Haemoglobin F (foetal haemoglobin) which is alkaline resistant, and Haemoglobin A (adult haemoglobin)

In the last decade a number of haemoglobin variants have been described The first of these variants to be described was Haemoglobin S which is found in 'sickle cell anaemia' The others have been labelled Hb-C, Hb-D, Hb-E, Hb-G, Hb-H, Hb-I, Hb-J, etc They are all permanent and inherited and it should be realized that they are all due to differences in the globin. Their biochemical characteristics are set out in Table I (at end).

In addition, Haemoglobin A has been analysed by new techniques and minor components have been demonstrated Next to the major fraction $A_1$ small amounts of two other fractions, called $A_0$ and $A_2$, have been discovered

In some haemoglobinopathies, all the haemoglobin is in one abnormal form, in others two abnormal haemoglobins may be present, in still others normal and abnormal haemoglobin are found together

Furthermore, in a number of cases foetal haemoglobin, in stead of normal adult haemoglobin, is present together with an abnormal hae-

moglobin, in a majority of these the carrier has inherited a thalassaemia gene from one of his parents.

In cases of thalassaemia major, foetal haemoglobin is usually greatly increased. In most cases of thalassaemia minor there is an increase in the Hb-$A_2$ fraction. In other cases there is an increase in the percentage of foetal haemoglobin.

It appears from all this that the synthesis of each abnormal haemoglobin is controlled by a specific gene. Some of these genes are likely to be alleles, but some combinations which are theoretically possible on this interpretation have not yet been found.

### DIAGNOSTIC METHODS

The major part of this manual is devoted to a detailed description of the haematological and physico-chemical methods used to detect the haemoglobinopathies. This chapter is therefore limited to a brief enumeration of the diagnostic steps to be taken in the elucidation of a case. These are as follows:

(1) Full history with particular reference to such symptoms as arthralgia and pain crises and to the occurrence of the same symptoms in other members of the family.

(2) Full clinical examination with particular reference to the size of the spleen and the liver.

(3) Estimation of the haemoglobin concentration (see p. 26).

(4) Total red blood cell count (R.B.C.) (see p. 28).

(5) Microscopic examination of blood films on slides (see p. 29).

(6) Determination of packed cell volume (P.C.V.), mean cell volume (M.C.V.), mean cell haemoglobin (M.C.H.), mean cell haemoglobin concentration (M.C.H.C.) (see p. 31).

(7) Reticulocyte count and determination of inclusion bodies in case of Hb-H (see p. 33).

(8) Determination of the osmotic fragility (see p. 35).

(9) Demonstration of sickle cells in the blood (see p. 37).

(10) Paper electrophoresis (see p. 12).

(11) Chromatography (see p. 18).

(12) Solubility determinations for differentiation between haemoglobins S and D (see p. 21).

(13) Alkaline denaturation tests (see p. 21).

*In all cases of haemoglobinopathies a complete investigation of all the members of the patient's family is desirable.*

## CLINICAL OUTLINE OF MAIN HAEMOGLOBINOPATHIES

The clinical and haematological features of human haemoglobinopathies are given in Table II (at end) In the following pages we shall only give a few clinical pointers to the various conditions

### 1. Conditions in which normal haemoglobin is replaced by one or two abnormal variants

#### Haemoglobin S

[illegible]

electrophoretic analysis Anaemia is often severe and tends to be relatively constant for a given individual, except for the so-called crises *In vitro* sickling tests show that practically all red cells sickle readily In the blood smear target cells are often observed in small numbers

M C.V, M C H and M C H C are generally normal, the red cells may be slightly macrocytic.

Sickling occurs as a result of relatively slight reduction of oxygen tension and initiates the cycle of increased viscosity, vascular stasis, obstruction, anoxia, ischaemia, necrosis, haemorrhage and thrombosis. These events account for the pain crises involving joints, bones, and muscles, abdominal pain, etc They account also for ischaemic necrosis of the bone, for the development of poorly healing leg ulcers, for infarction of the spleen and for functional and organic impairment of the liver The spleen is often enlarged in children, but later it tends to decrease in size and eventually may become very small Thickening of the skull may be observed in X-rays Where nutrition and medical facilities are poor, the majority of patients die in the first two years of life.

*Sickle Cell Trait* In the sickle cell trait all symptoms are far less severe Haemoglobin S accounts for less than 50% of the haemoglobin of the red cell, the rest being haemoglobin A. Under normal conditions sickling does not occur in the body On ordinary blood films it is not possible to detect any sickling Sickling may occur *in vivo* when the oxygen tension is lowered as is the case in high altitude flying and this may lead to splenic infarction *In vitro* it is possible to obtain sickling by reducing the oxygen tension Clinically the carriers are usually well Episodes of otherwise unexplained haematuria have been observed

*Sickle Cell Diseases.* Under this heading are grouped a number of diseases in which the sickling gene is present with another abnormal gene controlling haemoglobin synthesis. By order of decreasing clinical severity, these conditions are. sickle cell thalassaemia; sickle cell Hb-C disease, sickle cell Hb-D disease; sickle cell Hb-E disease; and sickle cell Hb-G disease.

### Haemoglobins C, D, E, G, I, J, K, L

*'Disease'.* This case represents the homozygous state. The red blood corpuscles contain solely the abnormal haemoglobin These conditions are associated with mild or minimal haemolytic processes and few symptoms Haemoglobin-C disease produces a mild haemolytic anaemia with a striking increase in the number of target cells, decreased osmotic fragility and marked splenomegaly. The only symptoms worthy of note are those related to the enlargement of the spleen The M C V, M C H and M C H.C. are usually normal

Other forms of the 'disease' appear to be nearly symptomless There may be a slight anaemia In the homozygous Hb-D and Hb-E disease the number of target cells is high and the osmotic fragility is decreased as in Hb-C disease In these latter diseases the M C V. and M C.H. have been found to be decreased, the M.C H C being normal.

*'Trait'* The trait represents the heterozygous state and therefore the red cells contain respectively haemoglobins A+C, A+D, A+E, A+G, A+I, A+J, A+K, and A+L These conditions appear to be harmless and anaemia, if present, is very mild.

In the Hb-C trait the number of target cells may be raised, in the other traits it is generally normal. The M C V. and M C H are sometimes slightly diminished in the Hb-E trait.

## 2. *Conditions in which there is an inherited defect in the production of Haemoglobin A*

### Thalassaemia

Thalassaemia was the first haemoglobinopathy for which a familial incidence was recognized In this condition there is an interference in the production of Haemoglobin A but there is no abnormal haemoglobin present.

*Thalassaemia Major or Cooley's Anaemia* This condition represents homozygosity for the abnormal gene and is characterized by a severe hypochromic microcytic anaemia without iron deficiency. There is

anisocytosis, poikilocytosis and many target cells (Mexican hat cells) Nucleated red cells are present when the anaemia is severe. After splenectomy the number of nucleated red cells may be very high Osmotic fragility is decreased and some red cells may even remain intact when suspended in distilled water. The M C.V , M C H and in many cases also the M C H C are decreased.

Although the blood picture resembles that of an iron deficiency anaemia, the iron content of the bone marrow is normal or high and iron therapy useless.

Haemoglobin F is nearly always present but the amount of this haemoglobin is not related to the severity of the disease.

Clinically, children affected are underdeveloped, presenting a mongoloid facies, splenomegaly and generalized bony changes due to hypertrophy of the marrow X-ray examination shows widening of the medullary portion of the bones, erosion and thinning of the cortex and a peculiar outline of the skull, often compared to hair standing on end. In typical cases, anaemia manifests itself some months after birth and life is rarely prolonged past childhood

*Thalassaemia Trait or Thalassaemia Minor.* This represents the hetero- [illegible]

cells The M C V and M C H are usually decreased, the M C.H C may be normal The osmotic fragility is decreased

There is usually an increased Hb-$A_2$ fraction but in other cases there is an increase in the amount of Hb-F of 5 to 10%. Occasionally thalassaemia minor presents as a relatively severe disease, and a genetic study is required to prove that it is not a case of thalassaemia major.

### 3. Conditions in which there is a combination of a thalassaemia minor and the heterozygous state for an abnormal haemoglobin

There are a number of combinations possible between thalassaemia and abnormal haemoglobin genes The combination of thalassaemia with an abnormal haemoglobin gene may suppress the formation of Haemoglobin A as effectively as homozygosity for the abnormal haemoglobin gene itself

[illegible]

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*Thalassaemia Trait or Thalassaemia Minor* This represents the heterozygous state and the majority of cases are relatively symptomless apart from a mild anaemia and polycythaemia with hypochromic microcytosis with ovalocytosis, poikilocytosis and a moderate number of target cells The M C V and M C H are usually decreased, the M C H C may be normal The osmotic fragility is decreased

There is usually an increased Hb-$A_2$ fraction but in other cases there is an increase in the amount of Hb-F of 5 to 10% Occasionally thalassaemia minor presents as a relatively severe disease, and a genetic study is required to prove that it is not a case of thalassaemia major

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Thalassaemia Hb-S disease is a well recognized entity and is the most severe condition clinically Each parent contributes an abnormal gene, for example the father contributing the thalassaemia gene and the

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# PHYSICO-CHEMICAL TECHNIQUES FOR THE IDENTIFICATION OF HUMAN Hb TYPES

## 1. Preparation of a haemoglobin sample

A heparinized blood sample of about 10 ml. is obtained by venepuncture. The plasma is removed as soon as possible. The remaining red blood cells are first washed three times with normal saline (0.9% NaCl) and three times with 1 25% saline and finally haemolysed by the addition of one volume of distilled water and 0.5 volume of toluene. The mixture is stored for 24 hours at 0-4° C. After centrifugation and removal of the toluene, the haemoglobin solution is shaken with silica-gel (Celite 535 or 545 or high flow supercel) and slowly filtered with suction on a Buchner funnel in order to remove the stroma proteins and some other impurities This purification procedure can also be achieved by centrifugation of the haemoglobin solution at 15000 r p m for 20-30 minutes For most analyses (except the estimation of the resistance to alkali denaturation) the haemoglobin is converted into mono-carboxy-haemoglobin by saturating the solution with carbon monoxide (This is prepared by adding small amounts of an 80% formic acid solution to concentrated sulphuric acid at about 60° C Precautions should be taken against exposure to carbon monoxide gas ) This CO-haemoglobin solution of 6-10 gm % Hb can be kept several weeks in a refrigerator without deterioration. In a frozen state it is even possible to keep the haemoglobin solution for several months without great changes Most analyses can also be carried out with oxyhaemoglobin It is then necessary to use a freshly prepared haemoglobin solution. The addition of a preservative (one drop of a 1:1000 solution of merthiolate or an antibiotic) is recommended

## 2. Sending of haemoglobin and blood samples

There are various ways of preparing samples for shipment to other investigators, depending on local possibilities.

(1) Specimens of solutions of oxyhaemoglobin (*NOT* CO-haemo-

mother the Hb-S gene. But it is possible for one of the parents to transmit the two abnormalities as they are genetically independent. The majority of patients with thalassaemia Hb-S disease presents a moderately severe sickle cell anaemia, the diagnosis being made by haematological methods. In many cases family studies are required to clinch the diagnosis.

Thalassaemia has been described in combination with Hb-C, Hb-D, Hb-E, Hb-G, and also with the association (Hb-G+Hb-S).

*Hb-H disease or Hb-H thalassaemia* Haemoglobin H has been found in patients with a disease which resembles a mild thalassaemia major The presence of this abnormal haemoglobin has not been demonstrated in either of the parents Generally one of the parents is a carrier of thalassaemia minor.

The presence of Haemoglobin H may be suspected if under certain conditions inclusion bodies are formed in the red cells

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London

shall therefore not discuss it in detail. Instead the reader is referred to the following general reviews on this subject

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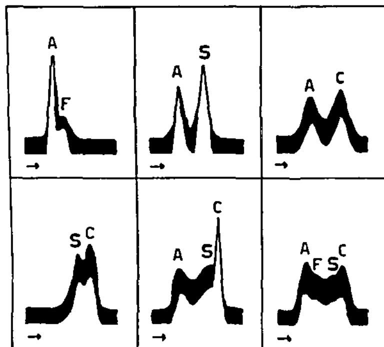



FIG 1

Moving boundary electrophoresis of mixtures of different human haemoglobin types

### 4. Zone electrophoresis on paper

A number of different procedures involving the use of filter paper electrophoresis have been employed with success for the detection and

globin as it may be necessary to estimate the resistance of the haemoglobin to alkaline reagents) are kept cold (0-4° C.) before and during the transport. They should be sent by airmail as soon as possible to the place of destination It is recommended that the tube be filled almost completely and that small amounts of a preservative be added (one drop of a 1 · 1000 solution of merthiolate or an antibiotic)

(2) A second method is to send washed red blood cells in a frozen state The erythrocytes are haemolyzed by this procedure and it is possible to prepare the haemoglobin solution at the laboratory upon arrival

(3) Washed erythrocytes are suspended in the Alsatian or ACD solution (mixture of citrate and glucose) and mailed by air. During transport the sample is kept in a refrigerator at 4° C.

(4) If there are no special facilities for washing at the place of collecting the blood samples, the red blood cells from uncoagulated blood are more or less freed from the plasma and sent by air without previous washings with saline. To prevent coagulation a small quantity of a mixture containing six parts of ammonium and four parts of potassium oxalate should be added to the blood; sequestrene, sodium citrate or heparin may also be used. As infection is one of the main causes of haemolysis one should try to get the blood sample as sterile as possible To get a sterile sample of blood the use of a so-called venule may be useful (manufactured by Bayer Products Ltd , 1 Africa House, Kingsway, London, W C.2). Venule 5 or 6 with Wintrobe solution or heparin should be used As the washing procedure has then to be carried out at the place of destination it is necessary to keep the erythrocytes at 0-4° C during transport and not in the frozen state It is not recommended to send specimens of whole blood

### 3. Moving boundary electrophoresis

The various known abnormal types of human haemoglobin have been discovered by the use of either moving boundary electrophoresis or filter paper electrophoresis The latter method has come into widespread use in clinical laboratories and will therefore be considered in some detail in the following section The moving boundary electrophoresis with the Tiselius apparatus has some advantages over other methods as it is possible with this technique to obtain quantitative data on mixtures of different haemoglobin types and also absolute mobilities. However, the method is not in general use for clinical laboratory work, and we

in 1 M $KCl$, or threaded wicks of cotton, placed through holes pierced in the baffle, provide electrical connection across the partition. The power supply recommended is that supplied by the E-C Apparatus Company. An inexpensive direct current power source may be obtained by removing the power pack from a salvaged radio (one in which separately mounted units are used as substitutes for battery operation).

*Reagents. Barbital buffer pH* 8.6, *ionic strength* 0 05

To obtain this, dissolve 20 6 gm of sodium barbital and 3.68 gm of barbital (diethylbarbituric acid) in distilled water Adjust the volume to 2000 ml.

*Phosphate buffer pH* 7.8, *ionic strength* 0 05

Dissolve 0 587 gm monobasic sodium phosphate ($NaH_2PO_4.H_2O$) and 6 50 gm. of dibasic sodiumphosphate ($Na_2HPO_4$, or 16 38 gm $Na_2HPO_4.12\ H_2O$) in distilled water Adjust the volume to 2000 ml.

*Phosphate buffer pH* 6 5, *ionic strength* 0 045

Dissolve 3 11 gm monobasic potassium phosphate ($KH_2PO_4$) and 1 49 gm of dibasic sodium phosphate ($Na_2HPO_4$ or 3 76 gm $Na_2HPO_4.12H_2O$) in distilled water Adjust the volume to 1000 ml

*Procedure*. Buffer solution is poured into the buffer compartments on the two sides of the apparatus. They are equilibrated for some time and the levels are adjusted on both sides to stand at about 1.5 cm under the top of the baffle.

A sheet (or a sheet partially cut into strips, or separate strips) of filter paper (Whatman No 3 or No. 3 MM, Schleicher and Schuell, No 598, Munktell No 20) cut slightly narrower than the plate dimensions is dipped into the buffer, blotted between layers of filter paper, and placed on the bottom plate. The glass plates are silicon coated, or Teflon sheets may be used between the plates and the paper sheet Rest the ends of the paper on the end walls of the apparatus and do not immerse them in buffer at this stage Apply about 10 µl of a 10% Hb solution to the paper in a straight line, working swiftly Another procedure is to apply the samples as spots (about 1-2 µl. of the Hb solution) Several samples may be applied to a single sheet of paper It is useful to include a control consisting of known Hb's with each run The ends of the paper are bent downward and immersed in the buffer solution The lid is closed and the clamps applied. A current of 250 to 300 volt is applied for 12-15 hours At the end of the experiment, the current is disconnected and

identification of abnormal human haemoglobins Two of these will be described, the first involving a pressure plate apparatus and the second a freely hanging strip

### 4.1. Electrophoresis with a pressure-plate apparatus

*Apparatus.* Different forms of this apparatus have been described some of which are manufactured commercially, for example the E-C

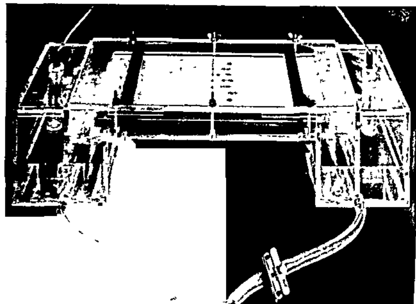


FIG 2
Pressure-plate apparatus for paper electrophoresis of human haemoglobin types

Apparatus Company, 23 Haven Avenue, New York They all employ two Plexiglass pressure plates (or plates of heavy window glass) of about 15 cm × 20 cm so arranged that cooling water can circulate through the plates This cooling system is not essential except in the tropics but it is desirable to work at relatively low temperatures (4-10° C.) Pressure is applied by means of adjustable clamps attached to the centre of the plates. The buffer compartments are divided into two independent sections The electrodes are platinum strips or carbon rods separated from the paper, which is inserted between the two plates, by a baffle Inverted glass U-tubes, filled with 3% agar made up

For most analyses the 0 05 M barbital buffer solution pH 8 6 is used; the relative mobilities of the different human haemoglobins at this pH value are as follows:

$$C<E<D=S<L<G<F<A<K<J<H=I$$

Semiquantitative data can be obtained by scanning the coloured strip in the same way as normally used for serum proteins.

### 4.2. Electrophoresis using a freely hanging strip

Different types of apparatus are used, they are manufactured by different companies (e.g Bender and Hobein, Munich, Germany,

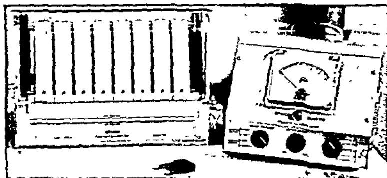


FIG 4

Hanging strip apparatus for paper electrophoresis of human haemoglobin types

A. H Thomas Company, Philadelphia, U S.A , and Beckman Instruments, Inc., Spinco Division, Belmont, Calif. U S A ) Strips of filter paper (Whatman 4 × 40 cm for instance) are used in any of these In principle the procedure is the same as that given for the pressure-plate apparatus The only difference is the relatively high evaporation rate to which the hanging strip technique is subject This sometimes influences development of the run

REFERENCES

BERC

GOL

ITAN

LARSON, D L., and RANNEY, H M (1953) Filter paper electrophoresis of human hemoglobins *J clin Invest*, 32, 1070-6

the lid is tilted carefully The filter paper is removed and allowed to dry at room temperature or is heated for some minutes. Although observation of the colour of the haemoglobin on the paper may often suffice for interpretation, staining in a solution of 0.1% bromphenol blue and

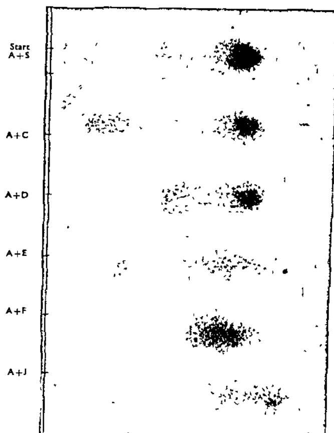



FIG 3

The separation of some human haemoglobin types by paper electrophoresis (Whatman 3 MM, barbital buffer pH 8 6)

10% mercuric chloride in 95% ethanol is more satisfactory. After 15 minutes in this solution, the paper is first heated at 120° C. for 5 minutes and immersed for 5 minutes in 2% acetic acid, followed by washing in water. The haemoglobin spots are blue against a normally white background.

For most analyses the 0 05 M barbital buffer solution pH 8.6 is used; the relative mobilities of the different human haemoglobins at this pH value are as follows.

$$C<E<D = S<L<G<F<A<K<J<H = I$$

Semiquantitative data can be obtained by scanning the coloured strip in the same way as normally used for serum proteins.

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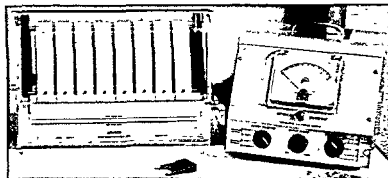


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Goldberg, C A

Itano, H A , Be
hemoglobins [illegible]

Larson, D L , and Ranney, H M (1953) Filter paper electrophoresis of human hemoglobins *J clin Invest* , 32, 1070-6

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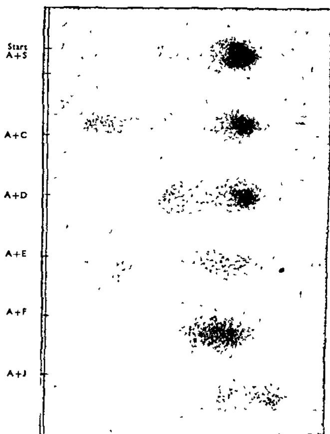



Fig 3

The separation of some human haemoglobin types by paper electrophoresis (Whatman 3 MM, barbital buffer pH 8 6)

[illegible] more satisfactory After 15 [illegible] 5 minutes [illegible] vashing in [illegible] hite back-ground.

block is 30 cm. long, 16 cm. wide and 3 to 5 mm. thick Relatively large samples of haemoglobin (0 05-0.1 ml. of a 10% solution) are usually applied to the slab by means of a capillary tube inserted in a slit of about 1 cm For the demonstration and quantitative estimation of the so-called $A_2$ component this amount is increased to 0 2 or 0.3 ml It is important that the starch be kept just dry enough to prevent fusion of

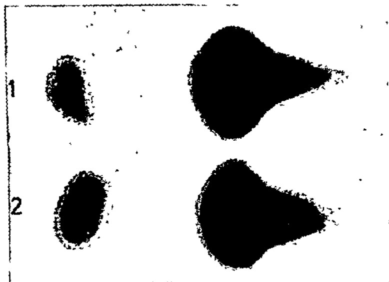



FIG 5
The estimation of the Hb-$A_2$ fraction (the small single component) in a normal adult (case 1) and in a Cooley trait (case 2) using starch slab electrophoresis (barbital buffer pH 8 6)

the slit Four to five samples can be applied to a single slab in this manner. It is also possible to absorb the Hb samples on small pieces of filter paper (Whatman 3 MM) and insert them into the slits. With a small spatula the starch is finally flattened at the sites of application When using a 0 05 ionic strength barbital buffer a current flow of 50-70 m amp with 300 to 350 volt (voltage measured between the two electrodes) is necessary

The resistance of this assembly is determined primarily by the molarity of the buffer solution and the thickness of the starch block.

The separation of the Hb-components is carried out over a period of

L[illegible] H. and S[illegible] E. G. [illegible]

M[illegible]

S[illegible]

S[illegible]

### 5. Zone electrophoresis on starch

This new method for the separation of larger amounts of haemoglobin and also for the demonstration of a heterogeneity of normal adult haemoglobin can be carried out in different ways. The apparatus and technique described below may be suitable for routine analyses in any average hospital laboratory

*Apparatus.* Two plastic boxes (20 cm. long, 10 cm. wide and 10 cm. high), divided lengthwise with a plastic partition, are used as buffer vessels. The central partition is pierced with five holes, just above the fluid level Through these holes are threaded wicks of cotton, connecting the fluid on either side of the partition The starch block is formed on a glass plate of 30 cm. length (direction of the current flow) and 16 cm wide. It is covered with a plastic sheet or with teflon. The electrodes are platinum threads or strips placed in the chambers at the ends of the apparatus A power supply by which a voltage of 300-500 volt can be obtained is recommended

*Reagents Barbital buffer solutions pH* 8 6, *ionic strengths* 0 05 *and* 0.10 prepared by dissolving 0 05 M sodium barbital and 0 02 M barbital or 0 1 M sodium barbital and 0 04 M barbital respectively.

*Starch* The kind of starch is important, as the amounts of calcium and of phosphate influence the separation of the haemoglobin components. The potato starch (reagent grade) manufactured by J. T Baker, Chemical Comp , U S A and the Amend potato starch will be found particularly useful. The material is washed over a filter with large amounts of buffer (not water) prior to use.

*Procedure.* A barely moist block of starch encased in a plastic layer is prepared first. For this purpose the starch, mixed with the buffer, is poured on to the glassplate covered with a thin sheet of clear plastic Blotting paper is used at the ends to confine the starch and absorb excess moisture. Weights are placed against the blotting paper to hold it in place. Contact of the starch block with the buffer solution is obtained by thick layers of filter paper over and under the starch. The starch

A hundred grammes of this resin is first stirred continuously with about 300 ml of distilled water for 15 minutes and allowed to settle for exactly 20 minutes. The supernatant is discarded This procedure is repeated four to five times, until the supernatant is clear. The air-dry resin is then stirred continuously with 300 ml of acetone for three hours

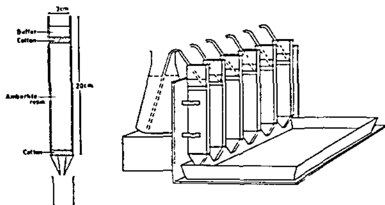



Fig 6

The equipment for the separation of different human haemoglobin types by chromatography on amberlite IRC-50

and finally washed with 1000 ml of acetone on a glass funnel. The resin is dried for a few hours at 50-60° C and washed with a large amount (3-5 litres) of distilled water in order to remove the last traces of acetone. The resin is then mixed with 500 ml 4 N sodium hydroxide, heated on a steam bath for ten hours and finally washed in a glass funnel with a large amount of distilled water (3-5 litres), until a neutral reaction is obtained In the same funnel 500 ml of 4 N hydrochloric acid are added to the resin and sucked through slowly. The excess of hydrochloric acid is removed by washing the resin again with 3-5 litres of distilled water. Finally the resin (100 gm ) is mixed with 50-70 ml of distilled water. It is possible to use the resin several times The regeneration procedure is the same as that given for the pretreatment of the resin except for the settling procedure and the treatment with acetone, which are omitted

*Citrate buffer solution pH* 6 0 1000 ml. of stock solution is prepared by dissolving 147 0 gm sodium citrate ($C_6H_5O_7Na_3$ $2H_2O$) in about 500 ml of distilled water. The pH of this solution is adjusted to 6 by means of a concentrated citric acid solution This solution is diluted 10 times before use.

approximately 15 hours It is necessary to work at low temperatures (4° C) *The position of each component is well recognized with the* aid of a light under the starch slab For quantitative estimation each fraction is outlined with a thin spatula, removed from the lower plastic sheet and packed on a ground glass filter. Water is then added to displace the haemoglobin from the starch A volume of water slightly greater than the volume of the packed starch is usually sufficient to remove all the haemoglobin. The relative concentration of the haemoglobin is finally obtained from readings in a Beckman spectrophotometer (or some other colorimeter) at 540 mμ The relative mobilities of the different haemoglobin types are the same as given by the paper electrophoretic technique

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KUNKEL, H G, and SLATER, R J (1952) Zone electrophoresis in a starch supporting medium *Proc Soc exp Biol (N Y)*, **80**, 42-4

KUNKEL, H G, and WALLENIUS, G (1955) New hemoglobin in normal adult blood *Science*, **122**, 288

It is also possible to estimate the $A_2$ content of a haemoglobin sample by electrophoresis on thick filter paper The technique is the same as that given in the section 'Zone electrophoresis in filter paper', except that the amount of haemoglobin applied to the paper is increased several times (see for instance Aksoy, M, *et al (1957) The recognition of* haemoglobins $A_2$ and E *Lancet*, 1, 792-3).

### 6. Chromatographic separation of haemoglobin types using the kation exchange amberlite IRC-50

*This technique is based upon the different absorption rates of the* human and also animal haemoglobin types It has been found possible to separate most abnormal haemoglobins from the normal adult haemoglobin The rates of displacement in kation exchange chromatography can be given as follows $C < L < S = D < G < E < A = J < I < F < H$ Haemoglobin K *has not been studied with this technique. Figure 8* presents a comparison between the results obtained by paper electrophoresis and Amberlite ICR-50 chromatography in the identification of some human haemoglobin types.

*Apparatus.* Flat lucite cuvettes measuring 20 × 3 × 0 5 cm inside filled with resin (see *below) and occluded at each end with cotton They* are placed on a rack. These cuvettes are manufactured by Pleuger Ltd, Antwerp-Brussels and Rotterdam

*Reagents Resin* Amberlite IRC-50 (XE 64) or (XE 97), 150-250 mesh, manufactured by Rohm and Haas Co, Philadelphia, Pa, U S A.)

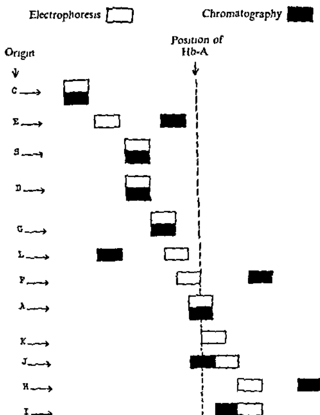



Fig. 8

The relative mobilities of different human haemoglobin types obtained by paper electrophoresis and Amberlite ICR-50 chromatography

*Procedure.* Each cuvette is filled to a depth of 15 cm. with the Amberlite resin. Equilibration of the resin is carried out with 25-50 ml. of the citrate-citric buffer pH 6 0 One-tenth ml of a haemoglobin solution (*containing about 10 gm % CO-Hb*) is diluted with 4 ml of distilled water and added to the surface of the resin. Equilibration of the *haemoglobin solution with the citrate buffer* has to be avoided, as in

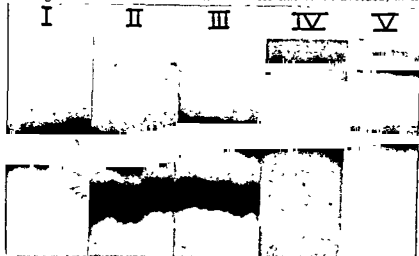



Fig 7
*Some examples of separation of human haemoglobin types by chromatography on* Amberlite IRC-50 I=Cooley trait, II=Cooley trait after addition of Hb-F, III=Cooley's anaemia, IV=sickle cell trait, V=heterozygous Hb-C disease

that case diffuse boundaries and bad separations of the haemoglobins are obtained Finally, the citrate buffer is siphoned from a supply bottle so as to pass through the cuvette at a maximum speed of 20 ml per hour A good separation of haemoglobins is made visible in 10 hours after about 200 ml of buffer have passed through the cuvette. It is recommended that these analyses be carried out at 4° C. It is possible to obtain semi-quantitative data on the percentage of the various haemoglobins in mixtures by measuring a diapositive of the cuvette. The range of error is 5 to 10%

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Huisman, T H. J, and Prins, H K (1955) Chromatographic estimation of four different human hemoglobins *J Lab clin Med*, 46, 255-62

methods available, based on this difference in resistance to alkali denaturation. We can divide them into two groups

## 1. METHODS

a *The spectrophotometric methods*, described by Brinkman and Jonxis (1935), also by Betke (1951) and Kunzer (1953, 1955) Recently Jonxis and Visser (1956) described a new variant, especially suited for the determination of low concentrations of foetal haemoglobin

The slow disappearance of the alkali resistant foetal haemoglobin is followed spectrophotometrically. This alkali-denaturation reaction of the foetal pigment is a first order reaction; it follows that the logarithms of the percentage of the undenatured haemoglobin plotted against time give a straight line. Extrapolation to zero time gives the amount of foetal haemoglobin in the sample

b. *The precipitation methods.* In these methods the haemoglobin (Singer, *et al*, 1951) or cyan-haemoglobin (Kunzer, 1953, 1955), exposed to alkali for some time, is precipitated and removed by filtration, after finishing the denaturation reaction by neutralization. The concentration of undenatured haemoglobin remaining in solution is measured spectrophotometrically

There seems to be general agreement that in both methods the denaturation of foetal haemoglobin follows first order kinetics For adult haemoglobin this is not the case. It seems to be likely that during denaturation with alkali the decrease in solubility of the denatured haemoglobin in a $33\frac{1}{3}\%$ ammonium sulphate solution and the corresponding change of the absorption spectrum do not take place at the same time This is perhaps the reason for the different results obtained by the two methods The precipitation method involves considerable error, particularly in the estimation of low percentages of foetal haemoglobin

## 2 REAGENTS, APPARATUS, PROCEDURE

a *Spectrophotometrical method of Jonxis and Visser*

0 06 N NaOH
10% $NH_4OH$

Beckman D.U spectrophotometer or its equivalent 0 1 ml. blood or haemoglobin solution (about 10 gm %) is diluted with 10 ml. of water to which 2 drops of $NH_4OH$ 10% are added The extinction of this solution is measured at a wave-length 576 mμ ($E_s$)

Next, 0.1 ml of the same blood is diluted with 10 ml 0 06 N. NaOH and 2 drops of $NH_4OH$ 10%. Each minute, especially between 5 and

### 7. The solubility of reduced haemoglobins

The low solubility of Hb-S in the reduced state distinguishes it readily from Hb-D which has a high solubility. This is of great importance for a differentiation as both Hb's have the same electrophoretic and chromatographic behaviour The only other haemoglobin known at the present time to have a low solubility in the reduced state is Hb-H, which shows a much higher electrophoretic mobility at pH 8 6 than Hb-A

*Reagents Phosphate buffer.* Dissolve 16 9 gm. monobasic potassium phosphate ($KH_2PO_4$) and 21 7 gm dibasic potassium phosphate ($K_2HPO_4$ or 17 7 $Na_2HPO_4$) in distilled water. Adjust the volume to 100 ml

*Solubility in 2 24 M phosphate.* Mix in a volumetric flask 8 0 ml of phosphate buffer and 100 mgr sodium hydrosulphite ($Na_2S_2O_4$) One ml of water is layered over this solution A volume of haemoglobin solution containing 50 mgr haemoglobin is added, care being taken to avoid precipitation of haemoglobin at this stage The flask is immersed in a water-bath at 25° C Finally, water is added to the 10 ml mark and the contents are completely mixed by inverting the flask several times If a precipitate appears the mixture is centrifuged at 17,000 × g. for twenty minutes Finally, the haemoglobin concentration of the solution is determined spectrophotometrically Under these conditions specimens containing Hb-S form an amorphous precipitate at a phosphate concentration of 2 24 M, while all other specimens dissolve completely A differentiation between sickle cell anaemia and sickle cell trait can easily be obtained by the estimation of the amounts of haemoglobin soluble in this phosphate mixture.

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GOLDBERG, C A (1957) Identification of human hemoglobins *Clin Chem*, 3, 1-19
ITANO, H A (1953) Solubilities of naturally occurring mixtures of human hemoglobins *Arch Biochem*, 47, 148-59

### 8. The alkali denaturation methods for the detection and estimation of foetal haemoglobin

Foetal human haemoglobin (Hb-F) has a much greater resistance to denaturation by alkali than other human haemoglobins This difference has found widespread application to the detection and estimation of foetal haemoglobin in the blood. There are now a number of estimation

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straight line By extrapolation to zero time the percentage of foetal haemoglobin in the original sample can be calculated

b *One-minute denaturation method of Singer*

$\frac{1}{12}$ N solution of NaOH or KOH kept in refrigerator, in paraffin lined bottles Precipitating solution 800 ml 50% saturated ammonium sulphate with addition of 2 ml 10 N hydrochloric acid

An approximately 10% haemoglobin solution is prepared from fresh oxalated blood obtained by venepuncture The cells are washed once with normal saline, shaken for five minutes with 1 2 to 1 8 volumes of distilled water (depending on the degree of anaemia) and 0 4 volumes of toluene, and the mixture centrifuged at 3000 r p m for twenty minutes The upper two layers are discarded, the clear red solution is filtered and adjusted to a concentration of approximately 10% by adding distilled water The exact haemoglobin concentration is then determined by one of the usual methods (dilution 50 times) One and six-tenths ml of the alkaline reagent is placed in a serologic test tube and kept in a water bath at 20° C for several minutes One-tenth ml of the haemoglobin solution is then added, the pipette is rinsed six times and the tube gently shaken for ten seconds A stop watch is started at the moment the haemoglobin is introduced into the denaturing medium After exactly one minute, 3 4 ml of the precipitating solution is added, the test tube inverted six times and the mixture immediately filtered through a double layer of filter paper

The amount of the alkali resistant fraction remaining in solution is measured with a spectrophotometer (Beckman D U , Coleman or its equivalent) at 540 mμ and expressed as a percentage of the initial amount of haemoglobin

To obtain a greater volume of filtrate, which may be necessary because of the size of the spectrophotometer cuvettes, it is perhaps better to work with twice the amounts

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BEAVEN, G H , ELLIS, M , and WHITE, J C (1956) Identification of small propor-

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Jc

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15 minutes, the extinction is measured ($E_T$). Next, the solution is placed in a water bath at 37° C. for 15 minutes After cooling to room temperature the extinction is measured again ($E_E$). The quotient $E_a : E_L$ should be constant.

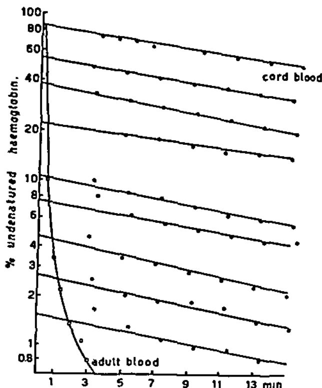



FIG 9
The alkali-denaturation curves of the haemoglobin of a normal adult, of cord blood and of blood samples of a child obtained at different times after birth (optical alkali denaturation method at 576 mμ, note that the slopes of the different lines are almost identical)

The percentage of the still undenatured haemoglobin at a certain time can be calculated as follows

$$\frac{E_T - E_E}{E_a - E_E} \times 100$$

Since the denaturation of the foetal haemoglobin occurs as a monomolecular reaction, the logarithms of the percentages of undenatured haemoglobin at different moments plotted against time will give a

## II

## HAEMATOLOGICAL STUDIES

### 1. Estimation of the haemoglobin concentration in blood or in haemoglobin solution

The haemoglobin concentration can be estimated by different methods In most of these procedures the haemoglobin is converted into other compounds, for instance acid haematin, alkaline haemochromogen or the reduced form The colour of the product is then compared with that produced by similar treatment of a blood sample of known Hb content, or even with an artificial standard Often, however, there are difficulties associated with the preparation and maintenance of a suitable standard Another complication is that most instruments used for comparing the colour of the sample with an artificial standard are calibrated differently When using one of these comparative procedures, it is necessary to indicate the instrument used and to give the equivalent of the standard in grammes of Hb per 100 ml of blood

The difficulties associated with the use of a standard are eliminated by a photometric calibration Using this principle, an accurate method, for instance, is a colorimetric alkali denaturation technique, measuring the alkaline haemochromogen with a suitable filter (for instance 640 mμ) The results should be standardized against a method based on the molar extinction of the Hb or on its iron content In this respect, a suitable procedure may be the cyan methaemoglobin technique, in which the extinction of cyan methaemoglobin of different samples at 544 mμ is compared with the known molar extinction coefficient In some countries (England, U S A , for instance) samples of cyan methaemoglobin have been distributed These standards are closely similar, international standardization is therefore possible

a *Alkali denaturation method*

REAGENTS 0 1 N sodium hydroxide solution 10% ammonia solution

APPARATUS spectrophotometer (Beckman D U or its equivalent) or colorimeter

PROCEDURE 0 1 ml blood is added to 10 ml 0 1 N sodium hydroxide with a few drops of 10% ammonia After some minutes (for adult haemoglobin, about five, foetal haemoglobin is more alkali-resistant

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SINGER, K, CHERNOFF, A I, and SINGER, L. (1951a) Studies on abnormal hemoglobins I Their demonstration in sickle cell anemia and other hematologic disorders by means of alkali denaturation *Blood*, 6, 413-29

SINGER, K, CHERNOFF, A I, and SINGER, L (1951b) Studies on abnormal hemoglobins II Their identification by means of the method of fractional denaturation *Ibid*, 6, 429-35

It is based on the same principle as method c. 0 02 C ml blood is diluted in an accurately measured volume of Drabkin's solution. In most photometers 5 ml of diluent are used The optical density of this solution is measured at 540 mμ and then compared with that of the standard

Drabkin's solution consists of $NaHCO_3$ 1 0 gm, KCN 50 mgr, $K_3Fe(CN)_6$ 200 mgr and distilled water to 1 litre

This is a clear solution, pale yellow in colour It should be discarded if it develops turbidity. It should be kept in a brown bottle and a fresh solution should be made up once a month

The concentration of cyanide in the reagent is so low that as much as 4 litres would be required to produce a lethal effect in man

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## 2. Total red blood cell count (R.B C.)

Red cells are counted in a counting chamber after dilution of the blood

MATERIALS 0 02 ml pipette (calibrated)

75 × 10 mm tube with tightly fitting rubber stopper

DILUTING FLUID 1% (v v.) formalin (40% formaldehyde) in a 3% trisodium citrate solution Pasteur pipettes

COUNTING CHAMBER

*Procedure* A dilution of 1 200 is made by pipetting 0 02 ml of blood into 4 ml of the diluting fluid, present in the glass tube The tube is sealed by its rubber stopper and the diluted blood is mixed for at least 2 minutes by tilting and rotating the tube, the air bubble serving to mix the contents

Next, the counting chamber with its cover glass in position is filled without delay, using a Pasteur pipette The counting chamber should be filled in one action, care being taken that the fluid does not reach the surrounding moat. The chamber should be left undisturbed for 3 minutes, allowing the cells to settle Counting should not be delayed longer, as drying at the edges makes the counting less accurate The generally used Burker-Turk and Neubauer counting chambers have a depth of 0 1 mm An area of 3 mm. is ruled and divided into squares and rectangles with sides of $\frac{1}{5}$ and $\frac{1}{20}$ mm The red cells, present in a certain area, are counted Cells overlying the top and left-hand margins should

and here the denaturation reaction is accelerated by placing the solution in a water bath of 37° C for 10 minutes) the red haemoglobin colour is changed into the brown colour of the denaturation product. The extinction coefficient of this solution is measured at 640 mμ. The corresponding haemoglobin concentration is calculated from a standard curve.

b *Reduction methods*

Reduce the haemoglobin with $Na_2S_2O_4$ (sodium dithionite) After addition of 0 04% ammonia, the extinction of the solution is measured spectrophotometrically at 556 mμ It is also possible to compare the colour of the sample with standards (Sicca)

REFERENCES

HEILMEYER, L, and VON MUTIUS, I (1938) Über die optische Bestimmung des Hämoglobins als Oxyhämoglobin, reduziertes Hämoglobin und Hämatin, Hämometerstandarisierung *Dtsch Arch klin Med*, 182, 164-75.

c *Cyan methaemoglobin method*

PRINCIPLE The haemoglobin is oxidized by potassium ferricyanide ($K_3Fe(CN)_6$) to methaemoglobin and this is transformed to cyanmethaemoglobin with potassium cyanide, giving a characteristic spectrum which remains stable for several hours

REAGENTS 0 1% KCN solution, 10% $K_3Fe(CN)_6$ solution

APPARATUS spectrophotometer

PROCEDURE 1 ml blood is mixed with 20 ml water Next, three drops of the potassium ferricyanide solution are added The oxidation is allowed to proceed for five minutes and 2 5 ml of the potassium cyanide solution is added to the mixture after which the volume is adjusted to 100 ml Extinctions are measured at wavelengths 540 and 544 mμ These values should be the same The concentration in gm per cent is obtained by the formula

$$C = \frac{E}{11\ 5} \times 16700 \times 10^{-4}$$

E = Extinction
C = Concentration in gm per cent
11 5 = millimolar extinction coefficient at both wavelengths
16700 = one-quarter of the molecular weight of haemoglobin

Another more simple cyan methaemoglobin method, using a standard, is the following one.

It is based on the same principle as method c. 0 02 C ml blood is diluted in an accurately measured volume of Drabkin's solution In most photometers 5 ml. of diluent are used. The optical density of this solution is measured at 540 mμ and then compared with that of the standard

Drabkin's solution consists of $NaHCO_3$ 1 0 gm , KCN 50 mgr., $K_3Fe(CN)_6$ 200 mgr and distilled water to 1 litre

This is a clear solution, pale yellow in colour. It should be discarded if it develops turbidity It should be kept in a brown bottle and a fresh solution should be made up once a month

The concentration of cyanide in the reagent is so low that as much as 4 litres would be required to produce a lethal effect in man

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## 2. Total red blood cell count (R.B.C.)

Red cells are counted in a counting chamber after dilution of the blood

MATERIALS 0 02 ml pipette (calibrated)

75 × 10 mm tube with tightly fitting rubber stopper

DILUTING FLUID 1% (v v ) formalin (40% formaldehyde) in a 3% trisodium citrate solution Pasteur pipettes

COUNTING CHAMBER

*Procedure* A dilution of 1 200 is made by pipetting 0 02 ml of blood into 4 ml of the diluting fluid, present in the glass tube The tube is sealed by its rubber stopper and the diluted blood is mixed for at least 2 minutes by tilting and rotating the tube, the air bubble serving to mix the contents

Next, the counting chamber with its cover glass in position is filled without delay, using a Pasteur pipette The counting chamber should be filled in one action, care being taken that the fluid does not reach the surrounding moat The chamber should be left undisturbed for 3 minutes, allowing the cells to settle Counting should not be delayed longer, as drying at the edges makes the counting less accurate The generally used Burker-Turk and Neubauer counting chambers have a depth of 0 1 mm An area of 3 mm is ruled and divided into squares and rectangles with sides of $\frac{1}{5}$ and $\frac{1}{20}$ mm The red cells, present in a certain area, are counted Cells overlying the top and left-hand margins should

and here the denaturation reaction is accelerated by placing the solution in a water bath of 37° C for 10 minutes) the red haemoglobin colour is changed into the brown colour of the denaturation product. The extinction coefficient of this solution is measured at 640 mμ The corresponding haemoglobin concentration is calculated from a standard curve

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PRINCIPLE *The haemoglobin is oxidized by potassium ferricyanide* ($K_3Fe(CN)_6$) to methaemoglobin and this is transformed to cyanmethaemoglobin with potassium cyanide, giving a characteristic spectrum which remains stable for several hours

REAGENTS 0 1% KCN solution, 10% $K_3Fe(CN)_6$ solution.

APPARATUS spectrophotometer

PROCEDURE 1 ml blood is mixed with 20 ml. water Next, three *drops of the potassium ferricyanide solution are added* The oxidation is allowed to proceed for five minutes and 2 5 ml of the potassium cyanide solution is added to the mixture after which the volume is adjusted to 100 ml Extinctions are measured at wavelengths 540 and 544 mμ These values should be the same The concentration in gm per cent is obtained by the formula

$$C = \frac{E}{11\ 5} \times 16700 \times 10^{-4}$$

E = Extinction
C = Concentration in gm per cent
11.5 = millimolar extinction coefficient at both wavelengths
16700 = one-quarter of the molecular weight of haemoglobin

Another more simple cyan methaemoglobin method, using a standard, is the following one

it The blood will spread quickly along the line of contact of spreader and slide. Next, the film is spread by moving the spreader quickly over the slide to the left

The thickness should be such that in the first part of the film there is some overlap of cells whereas towards the end the cells are separated and undistorted The length of the film should not exceed 3-4 cm.

### *Staining of blood film*

Generally Romanowsky stains are used in many modifications They consist of a mixture of methylene blue and eosin (Jenner, May-Grünwald) to which methylene blue azure may be added (Giemsa, Wright)

Methylene blue, which is a basic dye, will stain the acidic substances of the cells, whereas the basic substances will have an affinity for the acidic dye eosin The resulting colours depend mainly on the pH of the fluids used A pH of 6 8 is recommended Some prefer a higher pH (7 0 or 7 2) by which the blue colour produced by methylene blue will be accentuated Staining by eosin on the contrary is more pronounced at a lower pH

STAINING SOLUTIONS

The *Jenner or May-Grunwald staining solutions* are obtainable ready for use

The *Giemsa staining solution* is obtainable as a concentrated stock solution Immediately before use it has to be diluted (1 to 2 drops added to 1 ml of the buffer solution).

The *Wright staining solution* is obtainable ready for use

*Buffer solution* Generally the Sorensen phosphate buffer is used It may be prepared as follows·

*a* $\frac{1}{15}$ M $KH_2PO_4$ solution. Dissolve 9 078 gm $KH_2PO_4$ in 1000 ml of distilled water

*b* $\frac{1}{15}$ M $Na_2HPO_4$ solution. Dissolve 11.876 gm $Na_2HPO_4 \cdot 2H_2O$ in 1000 ml of distilled water.

From these two solutions a buffer with a pH of 6 8 is prepared.

PROCEDURE

*Jenner-Giemsa Stain* Place the slide for 2 minutes or longer in a staining jar, containing the Jenner solution Allow the Jenner solution to run off and cover the slide by the freshly prepared Giemsa dilution

be included, cells overlying the bottom and right-hand margins omitted. Areas to be counted should be chosen in different parts of the counting chamber.

*Calculation.* If in a Neubauer's counting chamber N red cells have been counted in four 1 mm. × 0 05 mm. rectangles (surface area 4 × 1 × 0 05 = 0 2 sq mm , depth 0 1 mm ; volume 0.02 c.mm ) in a dilution of 1 200, the total red cell count per c.mm. is

$$N \times \frac{1}{0\ 02} \times 200 = N \times 10000$$

If in the Burker-Turk counting chamber N cells have been counted in three 3 mm × 0 05 mm. narrow rectangles, the volume is 3 × 3 × 0 05 × 0 1 = 0 045 c mm and, using the same dilution (1 200), the total red cell count per c mm is

$$N \times \frac{1}{0\ 045} \times 200 = N \times 4400$$

*Remarks The accuracy of the red cell count is not very high If more* accuracy is needed, counting of the cells in a larger area is recommended Special red cell bulb pipettes diluting the blood 1 100 are still much used The results are sometimes very inaccurate.

*Normal values*

men 4 5-6 5 million per c.mm.
women· 4 2-5 6 million per c mm
infants at birth (full term, cord blood) 4 0-7 5 million per c.mm
children (1 year) 4.5 million per c mm (mean value).
children (10 years)· 4 7 million per c mm. (mean value)

### 3. Preparation of blood films on slides

Like all glass ware, slides should be absolutely clean. As a spreader, a slide or a thick piece of glass is used, the edge should be absolutely smooth If the edge is rough the film will show ragged tails at its end, containing many leucocytes. The spreader should be narrower in [illegible] the film is made. When capillary blood [illegible]

immediately after collection A small drop of blood is placed 1-2 cm. from the right-hand end of the slide, the spreader is placed at an angle of 30-45° on the left of the drop and moved back to make contact with

potassium oxalate mixture, consisting of six parts ammonium oxalate and four parts of potassium oxalate (2 mg. per 1 ml. of blood). Potassium oxalate must not be used alone as it causes shrinkage of the red cells The blood is carefully mixed by repeated inversion until bright red in colour. The haematocrit tube is then filled at once from the bottom to the 100 mm mark, using a capillary pipette or its equivalent The tube is centrifuged at 3000 r p m for at least 30 minutes The height of the red cell column is taken as the P.C.V. On the top of the red cells a greyish-red layer of leucocytes and a thin creamy one of thrombocytes will form These layers are not included

REMARKS It will be noted that some plasma is always trapped between the red cells. As only comparative values and no absolute data are needed, haematocrit values are not corrected for this inaccuracy

*Normal values*

| | |
|---|---|
| Men | 40-54% |
| Women | 36-47% |
| Infants (full term, cord blood) | 44-62% |
| Children (1 year) mean | 35% |
| Children (10 years) mean | 37 5% |

### 5. Mean cell volume (M.C.V.)

The M C V can be calculated from the packed cell volume and the number of red cells per c mm

$$\text{M C V} = \frac{\text{PCV (in per cent)}}{\text{red cell count (in millions)}} \times 10 \text{ c } \mu$$

### 6. Mean cell haemoglobin (M.C.H.)

*The M C H can be calculated from the haemoglobin content of the* blood (in gm per 100 ml ) and the red cell count

$$\text{M C H} = \frac{\text{Hb (in gm per cent)}}{\text{red cell count (in millions)}} \times 10 \ \mu\mu\text{g}$$

### 7. Mean cell haemoglobin concentration (M.C.H C.)

The M C H C can be calculated from the haemoglobin concentration of the blood (in gm per 100 ml ) and the packed cell volume

$$\text{M C H C} = \frac{100 \times \textit{Hb (in gm per cent)}}{\text{P C V. (in per cent)}} \text{ per cent}$$

for 10-15 minutes The slide is cleaned by running water of neutral pH and dried in the air Instead of the Jenner solution, the May-Grunwald solution may be used.

*Wright's stain* The slide is covered with the staining solution After



Fig 10

Photomicrograph of a blood smear in a case of thalassaemia-Hb-E disease Note the large number of target cells

1-3 minutes the same amount of the buffer solution is added This is left in position for twice the time of the primary staining (2-6 min ) The excess of dye solution is washed away and the film allowed to dry

**4. Determination of packed cell volume (P.C.V.) or haematocrit value**

(*a*) A haematocrit tube, usually the Wintrobe tube, is employed Its length is 110 mm , calibrated with 1 mm intervals to 100 mm and its internal diameter is approximately 3 mm

(*b*) A glass capillary pipette or long needle and syringe

PROCEDURE

Venous blood is taken with minimal stasis and rendered incoagulable by heparin (0 1-0.2 mg per 1 ml. of blood) or by a dry ammonium and

PROCEDURE One volume of the staining solution is diluted with five volumes of the citrate-saline solution

This diluted staining mixture may be used for weeks if the tube is kept closed.

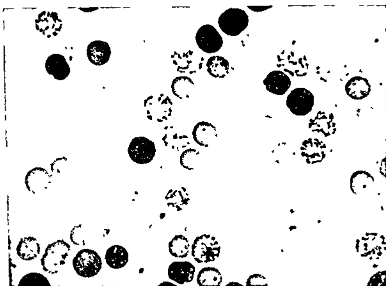


FIG 11
Reticulocytes in a case of thalassaemia major

One drop of fresh blood is mixed with one drop of the diluted staining mixture on a coated slide The resulting colour should be green If the colour is brown, more of the staining solution should be added, if it is blue, more blood The slide is placed in a moist chamber (Petri dish) at 37° C for 15 minutes The suspension is remixed with the rod and films are prepared The films keep well

*Normal values* 10-20 per 1000 erythrocytes.

*Haemoglobin-H inclusion bodies*

If Hb-H is present, inclusion bodies have been observed in many or in all of the red cells on incubation of the blood in a brilliant cresyl blue solution for 15 to 30 minutes Sometimes incubation for one or two

*Normal values*

| | |
|---|---|
| M.C V. | 76-96 c.μ |
| M C H | 27-32 μμg |
| M.C.H C. | 32-36% |

### 8. Reticulocytes

Usually 1000 red cells are counted and the number of reticulocytes noted and expressed as a percentage of the red cells. The counting is carried out under the microscope using an oil-immersion objective It is facilitated by providing the eye piece with a diaphragm. In this way a smaller field is visible in which the cells may be observed and counted with more accuracy The detection of riper reticulocytes, containing only a few dots or threads of reticular material, depends on the quality of the microscope and its resolving power and requires some training.

*The wet method*

The dye solution is spread on a slide and allowed to dry. A small drop of fresh blood is placed on a cover glass and this is placed, blood side downwards, on the stained slide The slide is placed in a moist chamber (Petri dish) at 37° C for 15-20 minutes. After this time the reticulocytes have taken up the dye and can be counted The preparation cannot be preserved

*The dry method of Seip*

MATERIAL

(*a*) 1000 mg. of brilliant cresyl blue and 400 mg of sodium citrate are dissolved in 100 ml of normal saline

(*b*) Hollow-ground slides, silicon (or paraffin) coated

(*c*) A small glass rod, silicon coated

PROCEDURE: 3-4 drops of blood are well mixed with an equal amount of the staining fluid in the hollow ground slide using the rod The slide is placed in a moist chamber (Petri dish) for 20 minutes Next, the stained blood is stirred with the glass rod and thin smears are made on slides. The preparations keep well for many weeks

*Dry method of de Vries*

MATERIAL

(*a*) A saturated alcoholic solution of brilliant cresyl blue.

(*b*) 1% solution of sodium citrate in normal saline

(*c*) Silicon or paraffin-coated slides and glass rod.

PROCEDURE: One volume of the staining solution is diluted with five volumes of the citrate-saline solution.

This diluted staining mixture may be used for weeks if the tube is kept closed

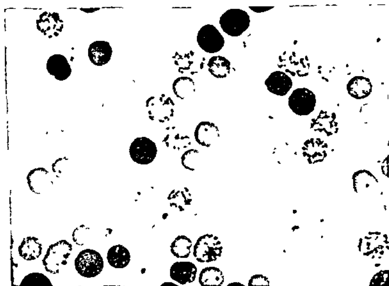


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*Normal values* 10-20 per 1000 erythrocytes

### *Haemoglobin-H inclusion bodies*

If Hb-H is present, inclusion bodies have been observed in many or in all of the red cells on incubation of the blood in a brilliant cresyl blue solution for 15 to 30 minutes. Sometimes incubation for one or two

hours may be necessary. Many small or a few larger refractile and rounded bodies will be observed, lying along the edge of the red cells. The inclusion bodies are not present in fresh blood or in Romanowsky stained preparations (Wright, Giemsa) and disappear on counterstaining with these dyes, whereas the reticular material in the reticulocytes retains its blue colour under these circumstances

PROCEDURES

(1) Two parts of blood and one part of a 1% brilliant cresyl blue solution in normal saline are incubated for 30 minutes to 2 hours at a temperature of 37° C. A small drop of the mixture is placed on a slide and examined under a coverslip

(2) On a slide one drop of brilliant cresyl blue solution is mixed with one drop of blood and covered with a coverslip The preparation is sealed with paraffin and incubated at 37° for 30 minutes to 2 hours.

### 9. Determination of the osmotic fragility

In this test the concentration of saline at which haemolysis is just detectable and the highest concentration of saline in which haemolysis appears to be complete are recorded

The osmotic fragility is markedly affected by

(*a*) the relative volumes of blood and saline

(*b*) the final pH of the blood-saline suspension and

(*c*) the temperature at which the test is carried out

It is important to use fresh blood as the fragility increases rapidly by storing It is therefore recommended that the results be compared with those of a blood sample taken from a normal person This blood sample should be taken and treated in exactly the same way as the patient's blood

MATERIAL

Small test tubes and pipettes

A solution of buffered sodium chloride, osmotically equivalent to 10% NaCl, prepared as follows

180 gm. of sodium chloride, 27-31 gm $Na_2HPO_4$ and 4 86 gm $NaH_2PO_4$ $2H_2O$ are dissolved in distilled water. The final volume is adjusted to 2000 ml.

PROCEDURE From the stock solution a 1% solution is made by dilution with distilled water Using this solution, test tubes are filled

with 5 ml of hypotonic dilutions, equivalent to 0 85, 0 75, 0 65, 0 60, 0 55, 0 50, 0 45, 0 40, 0 35, 0 30, 0 25, 0 20, 0.15, 0 10% NaCl It may be advisable to include intermediate concentrations The contents of the test tubes are mixed well before 0 05 ml (1 drop) of fresh heparinized blood is added They are mixed a second time and the red cells that are not haemolysed are allowed to settle at room temperature (20° C ), after which the results may be recorded In a hot climate it will be advisable not to wait until the cells have settled spontaneously but to centrifuge the suspensions after 30 minutes

If the tubes are placed in a refrigerator the fragility will be decreased Working at high or low (refrigerator) temperatures makes the use of a normal control sample a necessity

Instead of reading the results visually, one may measure the extinction of the supernatant fluid in a colorimeter at 640 mμ This offers the possibility of estimating the median corpuscular fragility (M C F, 50% haemolysis) which can be determined with more accuracy than the minimal and maximal fragility

NORMAL RANGE OF OSMOTIC FRAGILITY (at 20 C and pH 7 4)

| | | | |
|---|---|---|---|
| 0 30% NaCl | | 97-100% haemolysis | |
| 0 35% | ,, | 90- 99% | ,, |
| 0 40% | ,, | 50- 90% | ,, |
| 0 45% | , | 5- 15% | ,, |
| 0 50% | ,, | 0- 5% | , |
| 0.55% | , | 0% | ,, |

Median corpuscular fragility (M C F ) 0 40-0 445% NaCl.

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hours may be necessary. Many small or a few larger refractile and rounded bodies will be observed, lying along the edge of the red cells. The inclusion bodies are not present in fresh blood or in Romanowsky stained preparations (Wright, Giemsa) and disappear on counterstaining with these dyes, whereas the reticular material in the reticulocytes retains its blue colour under these circumstances

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PROCEDURE. From the stock solution a 1% solution is made by dilution with distilled water. Using this solution, test tubes are filled

bate or perhaps even better *B. coli*. When this is done the sickle shape, or sometimes polygonal shape, develops.

(i) When a drop of *B. coli* suspension together with a drop of blood or erythrocyte suspension is placed on a slide isolated from air with melted paraffin, and placed in a wet Petri dish or in a cardboard box in an incubator for 15 to 30 minutes at 37° C., the sickling of the erythrocytes is almost certain to occur within a short period of time.

(ii) A drop of blood mixed with a drop of a fresh 2% solution sodium metabisulphite (sodium dithionite, $Na_2S_2O_4$) also develops a lowered oxygen tension, so that sickling of the red cells occurs when sickle cell haemoglobin is present. Sodium dithionite is now available in tablets of 0.2 gm. from which a fresh solution can be made daily; sickle cells appear after 30 minutes. It is desirable to repeat this test to get more definite results. Care should be taken to avoid drying out, especially in a hot climate. Great differences are found between the shapes of the sickle cells obtained from patients suffering from sickle cell anaemia or sickle cell trait. Also the rapidity and the degree of sickle cell formation is of importance. Vandepitte (1955) has described a method for the differentiation of SCA and SCT patients. He found with Emmel's method sickle cell filaments in the bone marrow preparations of SCA patients. They are never found in SCT patients.

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### 10. The demonstration of sickle cells in the blood

Emmel, in a classical study, demonstrated the appearance of sickle cells in a drop of blood placed between an object glass and a cover glass, isolated from the air with paraffin



FIG 12
Sickled red cells from a case of sickle cell anaemia

Hahn and Gillespie (1927) found that the number of sickled cells increased rapidly by withholding oxygen from the erythrocytes, when returned to an oxygen rich atmosphere the sickle cells regained a normal rounded shape This peculiarity of the erythrocytes in changing from the biconcave shape into the sickle shape when the oxygen tension is lowered is characteristic of the presence of sickle cell haemoglobin The phenomenon is due to the considerably decreased solubility of reduced haemoglobin S, which is about 2% of that of reduced haemoglobin A

The method of observing the formation of sickle cells in a stained blood film cannot be recommended as the procedure may cause the appearance of a false sickle shape as a result of destruction of erythrocytes

It is recommended that the oxygen tension of the erythrocytes be lowered by means of oxygen-removing substances such as sodium-ascor-